CONTRIBUTIONS TO OUR KNOWLEDGE OF THE ACTINOMYCETALES. III.

Further Observations on the Genus Micromonospora.

By H. L. Jensen.
Macleay Bacteriologist to the Society.

(One Text-figure.)

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In a recent paper (Jensen, 1930) the present writer described 10 strains of Actinomyces-like organisms of a type for which Ørskov (1923) had previously suggested the generic name *Micromonospora*. It was not at that time found possible to classify them as definite species. The present paper represents a study of a larger number of strains, 67 in all, and in addition to these a culture of "*Streptothrix*" *chalceae*, the organism for which Ørskov suggested the name *Micromonospora*. This culture was obtained from E. Pribram's Mikrobiologische Sammlung (formerly Kral's Bakteriologisches Museum), Vienna.

"*Streptothrix*" *chalceae* was first isolated from the air by Foulerton (1905), who gave it a very brief description without any morphological details. In a later contribution, Foulerton (1910) shows macroscopic pictures of the cultures, but gives no further information. According to Foulerton and to a few notes by Leiske (1921), it is a long-hyphed actinomyces with a red vegetative mycelium and no aerial spores, possessing diastatic and proteolytic properties. Näslund and Dernby (1923) also state that it has a strong proteolytic power, but otherwise they do not comment upon it. Ørskov (1923) mentions the red colour of the mycelium in nutrient agar, besides a brownish-black discoloration in certain other media, e.g., water agar. He, as the first, describes its peculiar mode of spore formation: no aerial spores, but formation of small, oval, refractive, spore-like bodies situated singly at the tips of small branches of the vegetative mycelium.

The present strain of *S. chalceae* appeared to have undergone such profound changes during the long period of artificial cultivation, that it showed hardly any resemblance to the earlier descriptions. Macroscopically it produced a heavy, tough, wrinkled growth in agar media and potato, first whitish, later changing to dirty greyish-yellow, with no aerial mycelium or a trace of white. In liquid media it grew as small firm granules of the same colour, gradually forming a surface pellicle. On filter-paper in asparagine-solution it grew well, but without attacking the paper. Milk was slowly digested without any coagulation. Microscopical examination showed a tangled mass of richly branching hyphae, 0·4-0·7 μ thick, often with a granular content, but without any spores. Neither did the direct microscopical observation of the development of agar colonies (Ørskov, 1923) reveal any spore formation of the *Micromonospora*-type. Short filaments of aerial mycelium were occasionally seen in quite young colonies, but they disappeared again after 3–4 days without forming aerial spores. As this organism thus seems, since its examination in 1923 by Ørskov, to have lost the very

character upon which its identification first and foremost rests, viz., the spore formation, it cannot be identified directly with any of the other strains of *Micromonospora*.

Some of the 67 strains isolated by the writer were obtained from soil samples in the School of Agriculture, Sydney University; these are marked with numerals in Table I. Others were obtained from one particular soil (red clay from an orchard, of pH 7·9) with additions of cellulose or lignic acid; they are marked with capital letters followed by numerals. The medium for isolation was the dextrose-casein-agar previously described (Jensen, 1930); also the media for cultural study were the same as previously used. The temperature of cultivation was 30–32° C.

All strains are capable of liquefying gelatin and of hydrolyzing starch, although with varying rapidity. Nearly all digest milk,* mostly after a previous coagulation. Dextrose-asparagine-agar seemed of all solid media to give the most characteristic growth and spore formation. Nutrient agar and potato gave mostly an uncharacteristic growth consisting of a red, wrinkled mycelium without any spores. Morphological differences were found to be too slight to allow any real differentiation between the strains, but various cultural and physiological characters which might serve as a basis for species distinction, are listed in Table I. The constantly positive characters of diastatic action, digestion of milk and liquefaction of gelatin have not been included.

TABLE I.

Cultural and Biochemical Properties of Micromonospora.

Strain.	Colour of vegetative mycelium.	Sporulation.	Coagulation of milk.	Inversion of saccharose.	Reduction of nitrate.	Decomposition of cellulose.
P4	orange	very rapid	+	(+)	−	+
P3	,,	,,	+	(+)	−	−
C1	,,	,,	+	−	−	+
C2	,,	,,	+	+	−	+
C4	,,	,,	+	(+)	−	(+)
C7	,,	,,	+	+	−	+
L6	,,	,,	+	−	−	+
L7	,,	,,	+	−	−	+
125 VI	,,	,,	−	−	+	+
125 c	,,	,,	−	−	+	+
279 S3	,,	,,	−	−	+	+
163 III	,,	,,	+	−	−	−
176 XVII	,,	rapid	−	−	+	+
129 V	,,	,,	−	−	−	−
279 S1	,,	,,	−	−	+	(+)
L1	,,	,,	+	+	−	(+)
P1	,,	,,	+	+	−	(+)
U IV	,,	,,	+	+	−	+
P8	,,	,,	−	+	−	+
P13	,,	,,	+	−	−	+
P15	,,	,,	−	+	−	+
P18	,,	,,	+	+	−	+
C6	,,	,,	+	+	−	+

(+) indicates a faint reaction.

* A few strains did not show any digestion after two months, but they exhibited a proteolytic action on milk agar.

TABLE I—*Continued.*

Cultural and Biochemical Properties of Micromonospora.

Strain.	Colour of vegetative mycelium.	Sporulation.	Coagulation of milk.	Inversion of saccharose.	Reduction of nitrate.	Decomposition of cellulose.
C10	orange	rapid	+	+	−	+
L2	,,	,,	+	−	−	+
L3	,,	,,	+	−	−	+
L8	,,	,,	+	+	−	+
L11	,,	,,	−	−	−	+
P17	pink	,,	+	(+)	−	+
163 S1	orange	fairly rapid	−	−	+	−
P2	,,	,,	+	+	−	+
P6	,,	,,	+	−	−	+
P7	,,	,,	+	−	−	+
P12	,,	,,	−	−	−	+
L12	,,	,,	−	+	−	+
L14	,,	,,	+	(+)	−	+
L5	pink-orange	,,	+	(+)	−	+
P9	orange	slow	+	−	−	+
P10	,,	,,	+	(+)	−	+
P11	,,	,,	+	+	−	+
P14	,,	,,	+	+	−	+
P20	,,	,,	+	(+)	−	(+)
P21	,,	,,	+	+	−	+
P23	,,	,,	+	+	−	(+)
C5	,,	,,	+	+	−	−
C11	,,	,,	+	+	−	+
C12	,,	,,	+	+	−	−
L4	,,	,,	+	−	−	+
L9	,,	,,	+	+	−	+
L17	,,	,,	+	+	−	(+)
L18	,,	,,	+	+	−	−
L13	pink-orange	,,	+	−	−	(+)
P19	,,	,,	+	−	−	−
P5	,,	,,	+	−	−	+
176 XII	,,	,,	−	−	−	+
P22	,,	,,	+	+	−	−
C3	,,	,,	+	+	−	−
C8	,,	,,	+	(+)	+	+
U18	,,	,,	−	−	−	−
C9	pale pink	,,	+	(+)	−	+
125 II	brown	rapid	−	+	+	(+)
279 Sb	,,	,,	−	+	−	(+)
125 I	pale orange	very slow	−	−	−	−
279 S2	,,	,,	−	−	−	−
279 S4	,,	,,	+	−	−	−
176 IV	deep blue	very slow	−	−	−	−
125 a	,,	,,	+	−	−	−

(+) indicates a faint reaction.

It seems possible to distinguish only 4 broad groups of strains. Group 1, which is the largest, comprising 60 strains out of 67, includes strains which grow fairly rapidly on dextrose-asparagine-agar, producing a heavy, dense mycelium of various orange hues, mostly corresponding to "Light Salmon Orange", "Mikado Orange", "Orange Chrome", or "Cadmium Orange" (Ridgeway, Color Standards and Nomenclature). The intensity of the colour varies; it is often mixed with pink, and then often very pale or nearly white. Spores appear as a soft, glistening layer on the surface of the vegetative growth, of a colour corresponding to "Olivaceous Black", "Deep Slate Olive", or "Brownish Olive" (Ridgeway). In some strains the spore layer covers the whole surface within a week, and the dark colour spreads through the whole growth, but does not diffuse into the agar to any considerable extent. In other strains it may take a month or more to develop. There seems to be a certain association between pigmentation and abundance of spore formation, the strains with a pale pinkish-orange mycelium generally showing a slower sporulation. Most strains coagulate the milk previous to digestion, many of them invert saccharose, and some reduce nitrate to nitrite. When grown on filter paper, most strains are seen to attack the cellulose. The most actively cellulose-decomposing, rapidly sporulating strains (176 XVII, U IV, 125 c) produce a broad, greenish-black zone of disintegrated paper along the surface of the solution, others grow as extensive orange patches in which the paper appears corroded, softened and semi-transparent. The degree of attack also varies widely. There is no correlation between the four examined biochemical characters, which occur in no less than 10 different combinations (the most common of these is: coagulation of milk—inversion of saccharose—no reduction of nitrate—decomposition of cellulose), nor do these characters show any correlation with the pigmentation or the rapidity of sporulation. It seems necessary, therefore, to regard the whole group as one big "species-group" with a rather wide range of variation. *"Streptothrix" chalceae*, according to the data of previous authors (Foulerton, 1905; Lieske, 1921; Ørskov, 1923), seems to fall into this group with which it agrees in the following points: red vegetative mycelium without any aerial growth; no soluble pigments mentioned; comparatively strong proteolytic action (Näslund and Dernby, 1923). We will, therefore, at least until such a time when future study may show the possibility of further division of the group into more species, give it the name of *Micromonospora chalceae* (Foulerton), n. comb.

The second group (strains 125 II and 279 Sb) is distinguished from the first by the formation of a soluble pigment which first colours the initially orange mycelium deep brown to nearly black and afterwards diffuses into the medium, to which it imparts the same characteristic colour. The pigment is most distinct in dextrose-asparagine-agar or -solution, but very slight in gelatin or milk; it is thus different from the brown pigment produced by many species of *Actinomyces* in protein-containing media.* Sporulation is rapid and abundant, appearing as moist, brownish-black cushions on the surface of the growth. Milk is not coagulated, but slowly rendered semi-transparent, with a slight greyish-brown discoloration. Both strains invert saccharose, and one reduces nitrate to nitrite. They grow scantily on filter paper, producing small orange to brownish spots scattered over the whole strip of paper, which is not rendered transparent, but

* In one strain—125 II—the pigment has shown signs of becoming less intense after two years' cultivation.

slowly softened, so that after two months it can be reduced to a pulp by shaking the tube. This group seems sufficiently well defined to be regarded as a separate species, *Micromonospora fusca*, n. sp.

The third group (strains 125 I, 279 S2, and 279 S4) attaches itself closely to the least vigorously growing and most slowly sporulating strains of Group 1. The growth on dextrose-asparagine-agar is slow and scant, the vegetative mycelium is thin and flat, of a pale pink to pale orange colour, spreading deeply into the medium and not until lately showing thin greyish spots or crusts of spores. In milk they grow as small orange granules; one strain coagulates the milk and redissolves it slowly with a faint acid reaction. Saccharose is not inverted, nitrate is not reduced, and cellulose is not decomposed. This group may be called *Micromonospora parva*, n. sp.

Finally, strains 176 IV and 125a develop slowly, like those of the previous group, but eventually they produce a quite heavy and dense vegetative mycelium of a deep greenish-blue colour, most nearly corresponding to "Dusky Green Blue" (Ridgeway). The blue pigment does not diffuse into the agar. Its formation seems to depend on the free access of air; on the bottom of solution cultures the strains grow as round, firm granules of a white to pale pink colour. One strain coagulates milk, but the coagulum is not redissolved within two months, although a slight proteolytic action is discernible on milk-agar. Saccharose is not inverted, nitrate is not reduced, and cellulose is not decomposed. This group might properly be called *Micromonospora coerulea*, n. sp.

Summary Description of the Species of Micromonospora.

We are now in a position to give the following generic definition of *Micromonospora*: Actinomyces-like organisms, forming a mycelium of delicate, non-septate hyphae, 0·3–0·8 μ thick, without aerial mycelium (or traces, then without spores), but producing spores singly on the distal ends of short lateral branches of the vegetative mycelium; spores spherical to oval, 1·0–1·2 × 1·2–1·5 μ. Mycelium and spores Gram-positive, not acid-fast. Aerobic organisms, most frequently met with in soil. The type species is *Micromonospora chalceae* (Foulerton).

I. Vigorously growing organisms, typically with copious spore formation on dextrose-asparagine-agar.
   A. Vegetative mycelium pale pink to deep orange, no typical soluble pigment ..... *Micromonospora chalceae.*
   B. Vegetative mycelium orange changing to brownish-black, brown soluble pigment ..... *Micromonospora fusca.*

II. Slowly and feebly growing organisms, with scant spore formation on dextrose-asparagine-agar, no soluble pigment.
   A. Vegetative mycelium pale pink to pale orange .... *Micromonospora parva.*
   B. Vegetative mycelium blue ..... *Micromonospora coerulea.*

1. Micromonospora chalceae (Foulerton), n. comb.

Vegetative mycelium on dextrose-asparagine-agar heavy, compact, raised, not spreading much into the medium. Spore-layer well developed, moist and glistening, brownish-black to greenish-black, this colour sometimes spreading through the whole mass of growth. Grows in liquid media as small firm orange granules or flakes. Starch is hydrolyzed. Gelatin is liquefied. Milk is digested with a faintly acid reaction, mostly after a previous coagulation. Many strains invert saccharose. Some strains reduce nitrate to nitrite. Most strains decompose cellulose. Proteolytic action seems stronger in this than in the other species of this genus. Optimum temperature for growth, 30–35° C. Thermal death point of mycelium, 70° C. in 2–5 minutes. Spores resist 80° C. for 1–5 minutes.

2. Micromonospora fusca, n. sp.

Vegetative mycelium on dextrose-asparagine-agar heavy, compact, orange, rapidly changing to deep brown and nearly black; spore-layer moist, glistening, greyish- to brownish-black. Deep brown soluble pigment. Grows in liquid media as small brown granules and flakes. Starch is hydrolyzed. Gelatin is liquefied. Milk is slowly digested; no coagulation. Saccharose is inverted. Reduction of nitrate, positive or negative. Cellulose is attacked to a slight extent.

3. Micromonospora parva, n. sp.

Scant growth on dextrose-asparagine-agar; vegetative mycelium thin, spreading widely into the agar, almost colourless to pale pink or orange. Sporulation scant, giving rise to thin greyish, moist crusts on the surface. Starch is hydrolyzed. Gelatin is liquefied. Milk is left unchanged, or coagulated and slowly redissolved with faintly acid reaction. Saccharose is not inverted. Nitrate is not reduced. Cellulose is not decomposed.

4. Micromonospora coerulea, n. sp.

Slow growth on dextrose-asparagine-agar; vegetative mycelium dense, dark greenish-blue, with a hard and glossy surface. Sporulation very scant. The surface sometimes shows a thin white veil resembling aerial mycelium, but without aerial spores. Grows in liquid media as fairly large, firm, round, white to pink granules. Starch is hydrolyzed. Gelatin is liquefied. Saccharose is not inverted. Nitrate is not reduced. Cellulose is not decomposed.

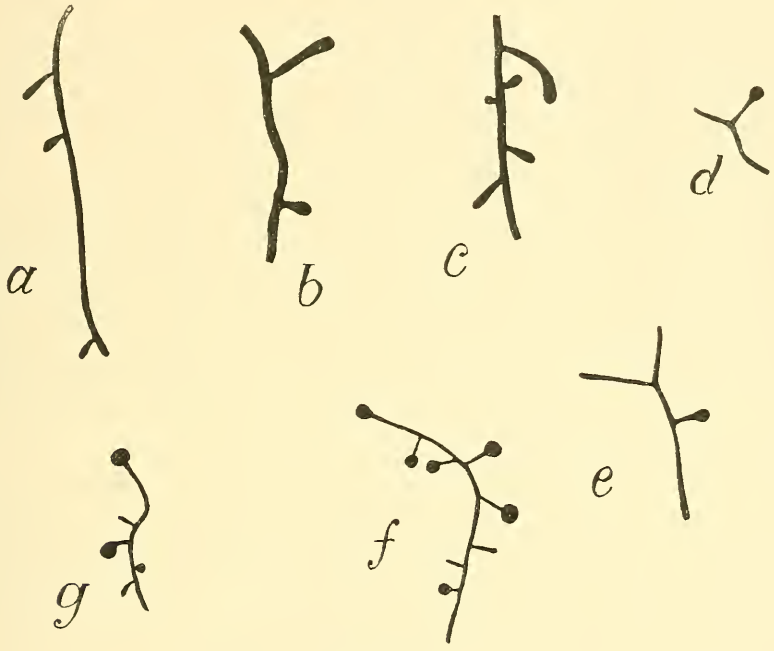
Relation of Micromonospora *to Other Genera of Actinomycetales.*

As to the relation of this genus to those other groups of Actinomycetales which it resembles most closely in its general appearance, viz., *Actinomyces* and certain forms of *Proactinomyces* (Jensen, 1931), it is noteworthy that there is a striking resemblance between the spore apparatus in *Micromonospora* and the formation of young mycelial branches in the other genera. As pointed out in a previous paper (Jensen, 1931), these branches arise as small external buds gradually stretching into filaments separated from the mother stem by basal constrictions and often assuming a club-like shape (Text-fig. 1, *a–e*). The conclusion lies near, that such club-like branches may further develop into those definite spores of reproductive capacity, which characterize *Micromonospora* (Text-fig. 1, *f–g*). Whether the ancestral forms of *Micromonospora* are to be sought among *Actinomyces* (in which case we would also have to assume a loss of the power of forming aerial spores in order to arrive at *Micromonospora*) or among forms of *Proactinomyces* with undivided vegetative mycelium (like the filamentous modification of *Proact. polychromogenes*, for instance), seems uncertain. It speaks for the former alternative, however, that *Micromonospora* in most biochemical respects agrees with *Actinomyces* rather than with *Proactinomyces*, as shown below:

	Micromonospora.	*Actinomyces.*	*Proactinomyces.*
Diastatic action	Constantly positive.	Constantly positive.	Positive or negative.
Liquefaction of gelatin	Constantly positive.	Constantly positive.	Positive or negative.
Digestion of milk ..	Constantly positive.	Constantly positive.	Positive or negative.
Decomposition of cellulose	Positive or negative.	Positive or negative.	Constantly negative.

Occurrence of Micromonospora *in Soils of Various Types.*

In a number of soils of various types, in which the numbers of bacteria and actinomycetes were determined by plate counts on dextrose-casein-agar, a special search was made for colonies of *Micromonospora*, which were identified by transfer to slants of dextrose-asparagine-agar. The results, recorded in Table II, show a certain evidence that these organisms are, at least relatively, most numerous in neutral to alkaline soils from districts with a low rainfall.



Text-figure 1.

a-c, Vegetative mycelium of *Actinomyces*; *a*, *Act. bobili*, dextrose-asparagine-agar, 2 d. 28° C.; *b*, *Act.* 6 S1, dextrose-casein-agar, 1 d. 28° C.; *c*, *Act. viridochromogenus*, 2 d. 30° C.; *d-e*, *Proact. polychromogenes*, filamentous form, dextrose-asparagine-solution, pH 8·3, 9 d. 30° C.; *f*, *Micr. coerulea*, filter paper, 18 d. 32° C.; *g*, *Micr. chalceae*, dextrose-asparagine-solution, 6 d. 32° C. Magnification, × 1,500.

SUMMARY.

A study of 67 strains of *Micromonospora*, isolated from soil, showed that these organisms exhibited only few morphological and biological differences which could be used for classification. On the basis of the character of growth in agar media and certain physiological differences, it was found possible to divide them into four species-groups, one of which is probably identical with "*Streptothrix*" *chalceae* (Foulerton). The other three are described as new species, *Micromonospora fusca*, *parva*, and *coerulea*. The first species (*M. chalceae*) was by far the most common. They seem to occur most frequently in neutral to alkaline soils from comparatively dry districts.

TABLE II.

Relative Frequency of Micromonospora *in Various Soils.*

Soil.	pH.	Average annual rainfall, i.*	Actinomycetes.	
			Total, Mill. per gm.	Micromonosporae %
Red sandy loam, poor in organic matter, Riverina..	7·9	15·7†	13·9	4·7
Red sandy loam, poor in organic matter, Riverina..	6·8	15·7†	2·7	17·5
Red sandy loam, poor in organic matter, Riverina..	6·5	15·7†	1·7	4·5
Red-brown sand, poor in organic matter, Cowra..	5·1	23·4	1·8	0
Alluvial clay, rich in organic matter, Bathurst ..	6·0	23·6	2·2	5·3
Humus soil, very rich in organic matter, Glen Innes	5·3	31·3	26·1	0
Heavy loam, rich in organic matter, Sydney University	7·3	47·5	2·9	0
Heavy loam, rich in organic matter, Sydney University	6·6	47·5	6·5	1·0
Heavy loam, rich in organic matter, Sydney University	5·5	47·5	3·2	0
Heavy loam, rich in organic matter, Sydney University	4·8	47·5	5·4	1·5
Sand, rich in undecomposed plant residue, Rose Bay	4·8	47·5	0·24	0

*From New South Wales Statistical Register, 1929–1930.

†Average of 3 stations in the Riverina District.

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